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Diversity of Endophytic Fungi in Lianas–*Combretum roxburghii* from West Medinipur District and its Seasonal and Regional Variation

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Abstract To determine diversity of endophytic fungi associated with the lianas *Combretum roxburghii* from different forests of West Medinipur district. Samples were collected randomly in various seasons. Fungi were isolated and identified based on mycelial shape and structure ; sexual and asexual reproductive characters ; attachment of spores and cultural conditions. A total of 603 endophytic fungi were isolated from 675 different sample segments. Colonization frequency is 74.66%. In Belpahari highest number of endophytes have been isolated and Chilkigarh was also nearer to Belpahari. In summer highest percentage (92.44%) were isolated. Dominant endophytic fungi were *Verticillium*, *Pestalotiopsis*, *Beltrania*, *Chaetomium*. Maximum endophytic isolates were obtained from stem segments followed by leaves and petioles. Among all endophytic fungi class Deuteromycetes were dominant over other fungi classes. Shannon-Weiner and Simpson's indices showed rich diversity of endophytic fungi. This indices suggest even and uniform occurrence of various species.

Keywords Endophytes, Lianas, Diversity, *Combretum*, Sacred grooves.

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Introduction

The term endophyte was first introduced by Anton De Bary in 1866, to describe organisms that live inside the plant. Huge number of endophytes remain in association with shade and moisture-loving woody climbers in stand forest [1]. The term includes all organisms that live symptomlessly within plant tissues at least for some period of their life cycle. The most commonly used and accepted definition is all organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host. Plants serve as reservoir of innumerable microorganisms known as endophytes [2, 3]. The most common usage of the term endophyte for those organisms whose infections are internal and inconspicuous and in which the infected host tissues are at least transiently symptomless, is equally applicable to bacterial prokaryotes and fungal eukaryotes [4]. Several recent studies have explored relationships between endophytes and their role as saprobes [5]. It seems likely that many saprobs are derived from endophytes [5]. Currently endophytes, which live in healthy plant tissues, are viewed as a wellspring of novel secondary metabolites offering the potential for medical, agricultural and / or industrial exploitation. Recently, many endophytic bioactive metabolites known as well as new substances, possessing a wide variety of biological activities as antibiotic, antitumor, anti-inflammatory, antioxidant have been identified. Chen et al. [6] isolated cytochalasins from cultures of an endophytic fungus. Many symbiotic endophytic fungi have been

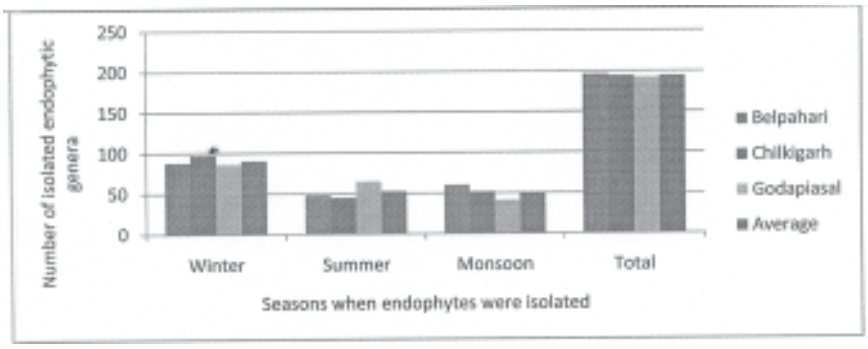

Fig. 1. Comparison of isolated endophytic fungi in three seasons from three localities.

isolated from three plants of family Lamiaceae [7]. Fungi are present in the intercellular-space of leaves, petioles and inner tissues of stems. They play a vital role in phosphate uptake in plant nutrition. It is well known that endophytic diversity is shaped by the host identity [8] and also known that the endophytic community depends on geographic situation [9] and seasonal variation. Only relatively few herbaceous plants and shrubs have as yet been examined till now. For the presence study of endophytes and study of its seasonal and regional variation an woody lianas plant *Combretum roxburghii* has been selected.

Materials and Methods

The study was conducted in West Medinipur district of West Bengal, India. The district is situated in between the latitude of 22°25´ to 22°57´ North and longitude of 87°11´ East. The altitude is 23M above from the sea level. The climate is tropical, warm and humid with a mean temperature of 33°C and an average rainfall of 120 cm. Lianas plant *Combretum roxburghii*, Family–Combretaceae, was selected. Plant samples (leaves, stems, petioles) were collected randomly from mature, healthy, disease-free plants from each location during winter, summer and monsoon. The samples immediately after collection were kept in zipper-lock plastic bags, brought to the laboratory and stored at 4°C within 2–3 h of collection untill isolation procedure was accomplished. Samples collected from different localities were thoroughly washed under running tap water before processing and following sequences were followed: leaf, petiole and stem samples were surface sterilized by sequentially dipping into 80% ethanol for 1 min, 1% sodium hypochlorite (NaOcl) (4% available chlorine) for 4 min, 90% ethanol for 20 sec. Finally, samples were rinsed with sterile distilled water for 3 times, then allowed to surface dry under sterile condition. Sterile leaves and petioles were cut into pieces of about 1 square cm size by sterile scissor and placed in plate of water agar (WA). Stem tissues were cut into short pieces after sequential sterilization, after removal of outer layer with sterile scalpel, thin peels from various depth were placed on another WA plate. Within 2–3 days fungal hyphae were in appearance. From each sample fungal hyphae were isolated and transferred to PDA media by cut-

Table 1. Colonization frequency of endophytes in various organs of *Combretum roxburghii* in winter at three places.

Place	Total segments plated	Segments infested with fungi	Fungi isolated from the segments	Colonization frequency (CF%)	CF% in leaf	CF% in petiole	CF% in stem
Belpahari	75	61	67	81.33%	52%	92%	100%
Chilkigarh	75	55	61	73.33%	88%	84%	48%
Godapiasal	75	52	60	69.33%	84%	48%	76%
Total	225	168	188	74.66%	74.66%	74.66%	74.66%

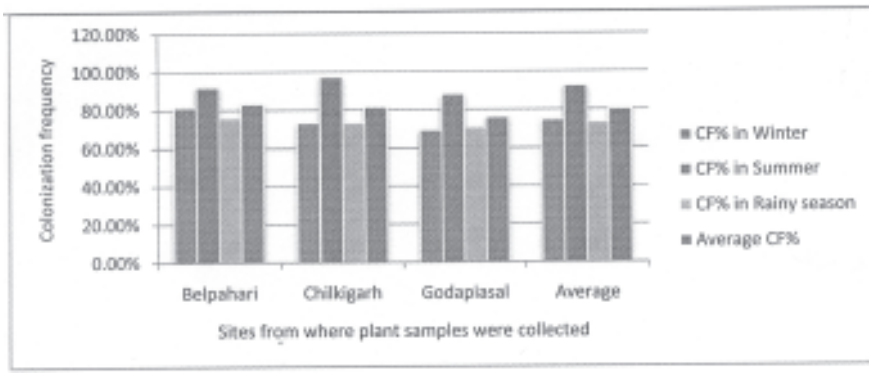

Fig. 2. Comparison of colonization frequency (CF) of isolated endophytic fungi.

ting a square block of water agar. The plates were incubated in light chamber at 23°C. After 10-15 days huge mycelial and in some cases reproductive growth was observed. Culture slants were made and preserved for identification at 4°C and also for further work in future. The endophytic fungal organisms were studied under optical compound microscope. The fungal isolates were identified based on their morphological and reproductive characters using the standard identification manuals.

Data analysis

The relative colonization frequency (CF%) was cal-

Table 2. Endophytic fungi isolated from leaf (L), petiole (P) and stem (S) segments of *Combretum roxburghii* from different localities during winter.

Isolated endophytic fungi	Total isolates	Belpahari			Chilkigarh			Godapiasal		
		L	P	S	L	P	S	L	P	S
Aspergillus sp.	09	0	0	0	3	1	3	0	0	2
Blastomyces sp.	01	0	0	0	0	0	0	1	0	0
Botryotrichum sp.	01	0	0	0	0	0	0	1	0	0
Chaetomium sp.	12	0	0	0	8	0	0	1	0	3
Chrysosporium sp.	04	0	0	0	0	0	0	4	0	0
Mucor sp.	02	0	0	0	0	2	0	0	0	0
Mycelia sterilia	11	1	0	0	5	4	0	1	0	0
Penicillium sp.	16	0	0	0	0	1	0	11	0	4
Podospora sp.	04	0	0	0	4	0	0	0	0	0
Scopulariopsis sp.	02	0	0	0	0	2	0	0	0	0
Tolyposporium sp.	03	0	0	0	0	0	0	0	0	3
Unidentified	49	0	0	0	5	14	1	9	11	8
Verticillium sp.	72	12	24	30	0	0	6	0	0	0
Zygorhynchus sp.	01	0	0	0	0	0	0	1	0	0
Total	186	13	24	30	25	24	10	29	11	20

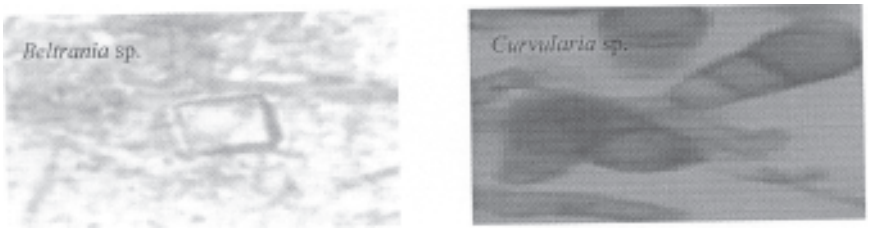

Fig. 3. Images of isolated endophytic fungi.

culated as the number of sample segments colonized by at least a fungus divided by total number of segments plated × 100 using the formula outlined by Hata and Futai : CF = (N_{col} / N_t × 100, where N_{col}) = number of segments colonized by at least a fungus, N_t = total number of segments plated. Using palaeontological statistics software package (PAST), following diversity indices were calculated:– (a) Simpson's Diversity Index (1-Dominance) was calculated using the formula 1-D, where D = Σn (n-1) / N (N-1). Here, n = the total number of organisms of a particular species, N= the total number of organisms of all species. (b) Shannon-Wiener diversity index was calculated using the following formula : Shannon – Wiener index (H´) = -Σ s (Pi) (In Pi), where H´ = Symbol for the diversity in a sample of species or kinds, s = the number of species in the sample, Pi = relative abundance of i^{th} species or kinds and measured by = n/N, N = total number of individuals of all kinds, n_i = number of individuals of i^{th} species, In = log to the base 2. (c) Evenness was calculated using the following formula : Evenness (E) = H´ / H´ max, where H´max is the maximum value of diversity for the number of species. (d) Species richness (S) is the simplest measure of biodiversity and is simply a count of the number of different species in a given area. This indicates the relative abundance of the taxa. (e) Fisher's Alpha index–It emphasizes the rate of species.

Results and Discussion

In winter study of *Combretum roxburghii*, out of 225 various tissue segments 168 segments had been colonized by endophytic fungi and 188 were isolated from them (Table 1, Fig. 1). Average colonization frequency was 74.66%. Leaf, petiole and stem i.e. all tissues showed equal colonization frequency and it was also 74.66% for each. But in stem of Belpahari CF was 100%. Out of isolated genera *Verticillium* sp. was highest in number (72) among total 188. *Penicillium* sp. and *Chaetomium* sp. were also good in number and it was 16 and 12 respectively. Total genera in

Table 3. Colonization frequency of endophytes in various organs of *Combretum roxburghii* in summer at three places.

Place	Total segments plated	Segments infested with fungi	Fungi isolated from the segments	Colonization frequency (CF%)	CF% in leaf	CF% in petiole	CF% in stem
Belpahari	75	69	69	92%	100%	88%	88%
Chilkigarh	75	73	73	97.32%	100%	100%	92%
Godapiasal	75	66	66	88%	80%	96%	88%
Total	225	208	208	92.44%	93.33%	94.66%	89.33%

Table 4. Endophytic fungi isolated from leaf (L), petiole (P) and stem (S) segments of *Combretum roxburghii* from different localities during summer.

Isolated endophytic fungi	Total	Belpahari			Chilkigarh			Godapiasal		
		L	P	S	L	P	S	L	P	S
Alternaria sp.	01	0	0	0	1	0	0	0	0	0
Arthrinium sp.	11	0	0	0	0	1	0	0	10	0
Beltrania sp.	01	0	0	0	0	0	1	0	0	0
Cochliobolus sp.	02	1	0	0	0	0	1	0	0	0
Diplodia sp.	01	0	0	0	1	0	0	0	0	0
Lasiodiplodia sp.	04	0	2	0	1	1	0	0	0	0
Mycelia sterilia	08	0	5	0	0	3	0	0	0	0
Nigrospora sphaerica	03	0	1	0	1	0	1	0	0	0
Pestalotiopsis sp.	98	17	15	10	16	8	13	1	3	15
Unidentified	04	3	0	0	1	0	0	0	0	0
Verticillium sp.	75	0	1	12	4	8	6	24	12	8
Total	208	21	24	22	25	21	22	25	25	23

Belpahari, Chilkigarh and Godapiasal were 2, 9 and 10 respectively. Dominance index was represented highest in Belpahari (0.9706). Simpson's diversity was maximum in Chilkigarh (0.8118) and Shannon–Wiener index was also highest (1.891) in plant of Chilkigarh. Here, the highest diversity of endophytic fungi was found in plants of Godapiasal. In summer study of *Combretum roxburghii*, out of 225 various tissue segments 208 segments had been colonized by endophytic fungi and the same number were isolated from them (Table 2, Figs. 2, 3). Average colonization frequency was 92.44%. Leaf, petiole and stem tissues showed colonization frequency of 93.33%, 94.66% and 89.33% respectively. It is obvious that highest CF was in plant of Chilkigarh. In leaf of Belpahari and Chilkigarh CF was 100% each and in petiole of Chilkigarh CF was also 100%. Out of isolated genera *Pestalotiopsis* sp. was highest in number (98) among total 208. *Verticillium* sp. was also greater in number (75). *Arthrinium* sp. was also good in number. Total genera in Belpahari, Chilkigarh and Godapiasal were 11, 3 and 6 respectively (Table 3, 4). Dominance index was represented highest in Godapiasal (0.4656). Simpson's diversity was maximum in Belpahari (0.6289) and Shannon–Wiener index was also highest in plant of Belpahari (1.4). From the graph it was

Table 5. Colonization frequency of endophytes in various organs of *Combretum roxburghii* in monsoon at three places.

Place	Total segments plated	Segments infested with fungi	Fungi isolated from the segments	Colonization frequency (CF%)	CF% in leaf	CF% in petiole	CF% in stem
Belpahari	75	57	89	76%	88%	64%	76%
Chilkigarh	75	55	64	73.33%	52%	88%	80%
Godapiasal	75	53	56	70.33%	48%	96%	68%
Total	225	165	209	73.32%	62.66%	82.66%	74.66%

Table 6. Endophytic fungi isolated from leaf (L), petiole (P) and stem (S) segments of *Combretum roxburghii* from different localities during monsoon.

Isolated endophytic fungi	Total isolates	Belpahari			Chilkigarh			Godapiasal		
		L	P	S	L	P	S	L	P	S
Arthrinium sp.	15	0	0	0	2	1	3	2	7	0
Aspergillus sp.	01	0	0	0	1	0	0	0	0	0
Beltrania sp.	24	12	0	2	2	3	1	1	0	3
Chaetomium sp.	24	1	1	1	4	4	2	2	8	1
Diplodia sp.	25	7	4	0	2	3	1	3	2	3
Fusarium sp.	19	0	5	9	0	1	1	2	0	1
Lasiodiplodia sp.	03	0	2	1	0	0	0	0	0	0
Mucor sp.	13	0	0	3	0	0	7	1	0	2
Mycelia sterilia	15	0	1	2	1	0	2	3	5	1
Paecilomyces sp.	01	0	0	0	0	0	1	0	0	0
Penicillium sp.	05	0	0	0	1	2	0	0	0	2
Pestalotiopsis sp.	46	19	0	5	3	8	7	1	2	1
Sordaria sp.	06	5	1	0	0	0	0	0	0	0
Unidentified	04	0	0	0	1	0	0	0	0	3
Verticillium sp.	08	3	4	1	0	0	0	0	0	0
Total	209	47	18	24	17	22	25	15	24	17

observed that maximum diversity of isolated endophytic fungi was in plants of Belpahari. In monsoon study of *Combretum roxburghii*, out of 225 various tissue segments 165 segments had been colonized by endophytic fungi and 209 endophytic fungi were also isolated from them (Tables 5, 6). Average colonization frequency was 73.32%. Leaf, petiole and stem tissues showed colonization frequency of 62.66%, 82.66% and 74.66% respectively. It is obvious that highest CF was in plant of Belpahari (76%) and in

Table 7. Diversity indices and species richness of endophytic fungi in *Combretum roxburghii* from Belpahari (Bel), Chilkigarh (Chil) and Godapiasal (Goda) during winter, summer and monsoon.

Parameters	Winter			Summer			Monsoon		
	Bel	Chil	Goda	Bel	Chil	Goda	Bel	Chil	Goda
Sp. richness	2	9	10	11	3	6	10	12	10
Individuals	67	59	60	68	73	65	89	64	56
Simpson diversity	0.02941	0.8118	0.7061	0.6289	0.5502	0.5344	0.8453	0.8521	0.8693
Shannon-Wiener index	0.07757	1.891	1.599	1.4	0.9278	1.08	2.049	2.145	2.152
Evenness	0.5403	0.7361	0.495	0.3688	0.843	0.4907	0.7761	0.7117	0.859
Fisher-alpha diversity	0.3877	2.959	3.427	3.716	0.6301	1.612	2.891	4.36	3.544

petiole (82.66%) of all localities. Out of isolated genera *Pestalotiopsis* sp. was highest in number (46) among total 209. *Diplodia* sp. (25), *Beltrania* sp. (24), *Chaetomium* sp. (24), *Fusarium* sp. (19), *Arthrinium* sp. (15) were also greater in number. Total genera in Belpahari, Chilkigarh and Godapiasal were 10, 12 and 10 respectively. Dominance index was represented highest in Belpahari (0.1547). Simpson's diversity was maximum in Godapiasal (0.8693) and Shannon–Wiener index was also highest (2.152) in plant of Godapiasal. From the graph it was clearly observed that the highest diversity of endophytic fungi was in plants of Chilkigarh. Average number of isolated endophytic fungi was highest in monsoon and in plants of Belpahari. But average colonization frequency was maximum in summer and in plants of Belpahari. Host-organ and tissue specificity has been observed in colonization of endophytes. There were diverse groups of fungal endophytes found in selected lianas plants in the study. Total isolated fungi in investigation during winter, summer and monsoon were 188, 208 and 209 respectively (Table 7). Fungi isolated from Belpahari, Chilkigarh and Godapiasal were 225, 198 and 182 respectively. Showed geographical and seasonal influences on the distribution of fungal endophytes. The endophyte diversity in different types of tropical forests of southern India is considerably lower when compared to that of the neotropics, perhaps owing to low floristic diversity, presence of relatively open canopies, highly variable annual rainfall and dry-season ground fires [10]. It was found that the highest colonization of endophytes was in summer. Belpahari showed highest isolation.

Conclusion

The infection and colonization of leaves and stems by endophytic fungi are affected by biotic and abiotic factors. Heterogeneity in physical microenvironments, such as sunlight intensity and moisture, which can lead to phenotypic changes in physical and chemical properties of leaves of plants, is expected to influence the colonization frequency of endophytic fungi. Endophytic fungal assemblages on leaves of plants in forest showed relatively minor seasonal and geographical changes. There were changes in colonization frequency in various seasons and different regions. In presence of higher water availability, nutrients can be available easily and in intense sunlight with sufficient moisture plant can add huge organic product which provide shelter and nutrients to endophytes. The lower variation in air temperature during the growing season (monsoon) in tropical forest may partly account for the less marked changes in fungal composition in different plants. Endophytic association of microorganisms with plants help in survival of both partners in adverse conditions of environment.

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